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Diphtheria Bacilli Isolated in Alberta in 1967 From the Throat, Nose, Ears and Skin

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IN recent years the incidence of clinical diphtheria in North America has greatly decreased. In Canada, 51 cases were reported in 1965[1] and 34 in 1966.[2] In the United States, 209 cases were recorded in 1966 and 219 in 1967.[3] Between August and December 1967, *Corynebacterium diphtheriae* was isolated at the Provincial Laboratory in Edmonton from 391 specimens submitted for diagnostic purposes from patients in northern Alberta and the adjacent Northwest Territories. Three hundred and sixty-one of the strains were lyophilized soon after they had been identified. In view of the rarity of occurrence of such a collection of strains on this continent at the present time, these organisms were studied in detail and their relationship to the clinical and immunological state of the patients was established when possible. The results of the study are presented in this paper.

METHODS

Isolation and Identification of C. diphtheriae

The specimens were swabs taken from the throat, nose and ears or from skin lesions and were received during the last six months of 1967 for routine bacteriological examination. They were cultured on 5% sheep blood agar and on Moore and Parsons' modification of Tinsdale's medium;[4, 5] the Tinsdale medium was inoculated by stabbing as well as by streaking the surface with the wire loop. Both media were incubated aerobically at 37° C. The identity of strains suspected of being *C. diphtheriae* was confirmed by their morphology in stained smears, their fermentation of dextrose and maltose in serum media, and their inability to ferment sucrose or to hydrolyze urea in Christensen's medium. The strains were classified as *gravis, intermedius* or *mitis* in type by their colonial appearance on McLeod's tellurite heated rabbit blood agar, their hemolytic reactions on sheep blood agar and their ability to ferment starch.

In vivo *Virulence Tests*

The overnight growth on a Loeffler coagulated serum slope was suspended in about 1 ml. of nutrient broth and 0.1 ml. was injected intradermally into the shaved abdominal skin of a white-haired guinea pig. Up to six cultures, appropriately spaced, were tested in each animal, which was then given 50 units of diphtheria antitoxin intraperitoneally. Identical inoculations were made into a control guinea pig that had received 500 units of antitoxin intraperitoneally on the day before the test. The sites of injection were inspected for erythema and necrosis daily for four days. A positive test was indicated by a necrotic reaction in the test animal but not in the control. In any doubtful instances, the test was repeated using 2 ml. of a suspension of an overnight serum slope culture injected subcutaneously into an unprotected guinea pig. Death within 96 hours with typical lesions at autopsy, but survival of a comparable animal that had received antitoxin, was regarded as a positive result.

In vitro *Virulence Tests*

An agar-diffusion method similar to that of Elek[6] was used, but the medium was that described by Hermann, Moore and Parsons,[7] which differs from most media used for toxigenicity tests in that it contains 0.033% potassium tellurite. The medium was poured into a 9-cm. diameter Petri dish. Before the agar had set, a strip of Whatman No. 1 filter paper measuring 7.5 x 1.5 cm. which had been soaked in a freshly prepared dilution of diphtheria antitoxin (Connaught Medical Research Laboratories, Toronto, Ontario) at a concentration of 200 units per ml. was placed on the surface of the agar and allowed to sink beneath the surface. (Previously tested antitoxin was used; there is a variation in the optimal concentration of different lots.) Plates were dried for 45 to 60 minutes at 37° C. and used within four hours of preparation.

Inoculation technique.—Organisms were inoculated by a 2-mm.-diameter wire loop in the form of a 2-mm.-wide streak across the plate at right

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angles to the paper strip. This width of inoculum gave optimal results. Each plate was inoculated with two test organisms and also with the following three organisms as controls: *C. diphtheriae minimus,* strain No. 14779 of the American Type Culture Collection (ATCC), which is weakly toxigenic; a strongly toxigenic *intermedius* strain isolated locally; and an atoxigenic strain of *C. diphtheriae* (ATCC 11913). The pattern of inoculation was such that each test organism was streaked next to the *minimus* control strain. Inoculated plates were incubated in sealed jars in a moist atmosphere of air at 36° C. The addition of carbon dioxide to the air was noted to be deleterious and retarded the formation of lines of precipitation.

Reading.—Plates were examined by the naked eye and by using a X3 lens in a strong oblique light against a dark background after 24, 48 and 72 hours' incubation. Tests were read when definite lines of precipitation appeared from *minimus* strain ATCC 14779. Plates were never read later than 72 hours after inoculation, and a check was always made to ensure that the non-toxigenic strain gave no reaction.

RESULTS

The origin of the 361 strains studied and the results of the toxigenicity tests are given in Table I. Twenty-two strains (6.1%) were from discharging ears or skin lesions. Of the 339 strains isolated from the nose or throat, 121 (35.4%) were toxigenic by the *in vivo* and *in vitro* tests used. With eight strains, four of *intermedius* and four of *gravis* type, the intracutaneous test gave a negative result while the *in vitro* test was positive. When the *in vivo* test was repeated by the subcutaneous route, the eight strains all gave definite positive results. Thus finally the results of the two tests agreed in every instance.

TABLE I.—DIPHTHERIA BACILLI ISOLATED IN 1967. EIGHT CLINICAL CASES OF DIPHTHERIA ARE INCLUDED; THE STRAINS ISOLATED FROM SEVEN OF THESE WERE TOXIGENIC, AND THE ONE FROM THE OTHER CASE WAS NON-TOXIGENIC.

Site of isolation	*Toxigenic*	*Non-toxigenic*	*Total*
Throat or nose	121	218	339
Ear	7	9	16
Skin	2	4	6
Total	130	231	361

The type distribution of the strains is given in Table II. Ninety-five (93.1%) of 102 *intermedius* strains were toxigenic, but only 34 (27%) of 126 *gravis* strains and one of 133 *mitis* strains produced toxin.

TABLE II.—CLASSIFICATION OF 361 STRAINS OF *C. diphtheriae* ISOLATED IN ALBERTA IN 1967

Type	*Toxigenic*	*Non-toxigenic*	*Total*
Gravis	34 (27.0%)	92 (73.0%)	126
Intermedius	95 (93.1%)	7 (6.9%)	102
Mitis	1 (0.8%)	132 (99.2%)	133
Total	130 (36.0%)	231 (64.0%)	361

A comparison of the *in vivo* and *in vitro* methods is shown in Table III, in which is recorded the time at which positive readings could be made in each test. The advantage of the *in vitro* test is clear from the 24-hour readings, at which time 81 (62%) of 130 *in vitro* tests were positive but none of those in the guinea-pig skin were readable. The value of the tellurite component of the medium was noted in a comparison with medium from which tellurite was omitted but which was otherwise identical. In many instances the lines of precipitation took longer to appear in the tellurite-free medium, and they never appeared earlier.

TABLE III.—RESULTS OF TOXIGENICITY TESTS ON 130 VIRULENT STRAINS

	Gravis		*Intermedius*		*Mitis*		*Total*	
Time of appearance of positive result	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
24 hours	0	18	0	62	0	1	0	81
48 hours	28	16	80	17	1	0	109	33
More than 48 hours	6*	0	15*	16	0	0	21	16
Total	34	34	95	95	1	1	130	130

*Four *gravis* and four *intermedius* strains gave negative results in the intracutaneous *in vivo* test but subsequently gave positive results in a subcutaneous test.

Of the 339 patients from whom diphtheria bacilli, including 121 toxigenic strains, were isolated from nose or throat swabs, only eight were regarded as cases of clinical diphtheria. In one of these patients the strain isolated was non-toxigenic by both *in vivo* and *in vitro* techniques.

Information was kindly made available by local medical officers on the state of immunization of 215 of the symptomless nose or throat carriers. The immunization state was classified as Full, Lapsed, Inadequate or None according to the definitions in use at the National Communicable Disease Center of the United States Department of Health, Education, and Welfare, Atlanta, Georgia.[8] Full immunization is defined as a primary series of three or more injections, or a primary series plus a booster, completed within four years of the onset of illness. In Table IV the immunization state has been related to the type and toxigenicity of the organisms iso-

TABLE IV.—DETAILS OF DIPHTHERIA BACILLI ISOLATED FROM THE NOSE AND THROAT OF 215 SYMPTOMLESS CARRIERS OF WHOM THE IMMUNIZATION HISTORY IS KNOWN

Immunization state	*Toxigenic*			*Non-toxigenic*			
	Grav.	*Int.*	*Mit.*	*Grav.*	*Int.*	*Mit.*	*Total*
Fully	0	58	0	50	2	64	174
Lapsed	2	5	0	18	0	5	30
Inadequate	0	0	0	0	0	2	2
None	0	3	0	6	0	0	9
Total	2	66	0	74	2	71	215

lated. Of the 215 carriers for whom information was available, 174 (80.9%) were fully immunized before the summer of 1967. Toxigenic strains were isolated from only 10 carriers who were less than fully immunized. The age distribution of the patients probably accounts in part for the excellent state of immunity: of 322 patients from whom diphtheria bacilli were isolated and whose ages were known, 37 were less than 5 years of age, 119 were aged 5 to 9 years, 79 were 10 to 14 years, 60 were 15 to 19 years and 27 were 20 or more years of age; 73% were under 14 years of age. Many of the children were Indian or Metis, and lived on reserves or in small rural communities.

DISCUSSION

From these results it is clear that despite high levels of artificially induced immunity, diphtheria bacilli, many of them toxigenic, are still widespread in children in certain parts of Canada. Fortunately the incidence of clinical disease is low, but the occurrence of these dangerous organisms on the scale that has been revealed by our studies emphasizes the importance of maintenance of immunity to a disease which many of the present generation of the general public may believe is one of the past.

The 22 strains isolated from the ears and skin may be of much greater epidemiological significance than the 339 strains from the nose and throat. Clearly the opportunity for dissemination of bacteria is much greater from an external site, particularly a discharging one, than from the nose or pharynx. The occurrence of diphtheria bacilli in cutaneous ulcers and other skin lesions in tropical countries has been known for many years, and was studied in particular during the two world wars in members of the armed forces.[9, 10] In a recent study of a semi-rural population in Ceylon, 664 children were examined and *C. diphtheriae* was isolated from swabs of 40 cutaneous ulcers and from six swabs of the skin of the legs;[11] the possibility was considered that these superficial skin infections may be a means whereby the population acquires immunity to diphtheria, thus preventing the occurrence of the faucial form of the disease. The results were reported recently[12] of a systematic search in Alabama and Louisiana for diphtheritic skin infections. In some of the areas studied clinical diphtheria had not occurred for eight years, but skin infections were detected. Thirty of 268 otherwise healthy persons harboured diphtheria bacilli in skin lesions, and there was some evidence that infection was transmitted from the skin to the respiratory tract. The two states in which this investigation was conducted have a subtropical moist climate. Little attention, however, has been paid to the possibility that a cutaneous reservoir of diphtheria bacilli may be an important epidemiological factor in a cold temperature of low humidity, such as prevails in Alberta. Flor-Henry[13] commented in 1961 that cutaneous diphtheria may be more prevalent in North American Indians than is generally recognized, but reported only two cases, which occurred in Hobbema, Alberta; a family contact of one case carried virulent diphtheria bacilli in the throat. The significance of our isolations from the skin and ear in 1967 is more easily assessed when the figures for 1968 are also considered. Forty-nine strains of diphtheria bacilli, including 22 toxigenic strains, were isolated at this laboratory from skin and ear lesions in 1968. Thus, in the years 1967 and 1968, 71 isolations of diphtheria bacilli were from external body sites, and 31 of the organisms were toxigenic. These findings indicate that diphtheritic infections of the skin and external ear may occur not uncommonly in climatic conditions very different from the tropical and subtropical regions from which most of the previous reports have emanated. Superficial skin lesions and external ear infections may be an important reservoir of diphtheria bacilli in Alberta. They may be a major factor in the continuing endemicity of diphtheria in the province despite a high level of artificial immunization.

We wish to mention two features of laboratory technique which concern work on diphtheria bacilli. The first is the value in our hands of the tellurite-containing medium of Hermann, Moore and Parsons[7] in the *in vitro* toxigenicity test. These authors noted the value of 0.033% tellurite in intensifying and accelerating the formation of the lines of precipitation in the *in vitro* test; we confirm this and recommend the routine use of this medium, which we found to be consistently reliable. Secondly, the use of modified Tinsdale's medium[4, 5] for the primary isolation of strains from swabs of nose, throat, ears and skin allows the naked-eye recognition of diphtheria bacilli by the brown halo formation that

results from the reaction of potassium tellurite with hydrogen sulphide which is produced by the bacilli from the L-cystine in the medium. The haloes may be visible in 24 hours but generally are not seen for 48 hours. The reaction also occurs with *C. ulcerans* but, in our experience, with no other organism likely by its colonial appearance to be confused with *C. diphtheriae*. Unfortunately, some variation in the performance of different batches of medium was noted, and each batch must be tested before use. The medium is useful in dealing with large numbers of specimens, such as those from contacts of a case in an institution, but characteristic colonies are, on the whole, slower to appear on Tinsdale's medium than on Hoyle's tellurite-lysed blood agar. An incidental observation made during this study was that incubation in an atmosphere of 5 to 10% carbon dioxide is deleterious to Tinsdale's medium and retards the development of the characteristic halo.

Summary In 1967, 361 strains of *C. diphtheriae* were isolated from specimens from the throat, nose, ears and skin of patients living in and to the north of the Province of Alberta; 130 (36%) were toxigenic, and 95 of these were of the *intermedius* type. Clinical disease was uncommon, probably because of the high degree of immunity of the persons examined, who were mainly school-children from rural areas.

Twenty-two strains were isolated from discharging ears or skin lesions, and the suggestion is made that such lesions may form an epidemiologically important reservoir of diphtheria bacilli in Alberta.

Toxigenicity tests were performed by *in vivo* tests in guinea pigs and by an *in vitro* agar-diffusion test using the medium of Hermann, Moore and Parsons. The *in vitro* test was shown to be very reliable and gave results more quickly than the animal test, 81 of 130 positive tests being readable after 24 hours' incubation. Its use is recommended.

Résumé En 1967, nous avons pu isoler 361 souches de *C. diphtheriae* dans des spécimens prélevés dans la gorge, le nez, les oreilles et la peau de malades vivant en Alberta et dans le nord de la province. De ces souches, 130 (soit 36%) étaient toxigènes et 95 d'entre elles étaient du type *intermedius*. Peu de cas cliniques, probablement en raison de la forte immunité acquise par les sujets examinés, qui étaient en ordre principal des écoliers des régions rurales.

On a isolé 22 souches dans le liquide d'écoulement des oreilles ou dans des lésions cutanées. On peut supposer que ces lésions peuvent constituer un important réservoir épidémiologique des bacilles diphtériques en Alberta.

On a procédé à des épreuves de toxigénicité *in vivo* sur des cobayes et une épreuve *in vitro* de diffusion sur agar, au moyen du milieu de Hermann, Moore et Parsons. Ce test *in vitro* s'est révélé très sûr et a donné des résultats plus rapidement que l'épreuve biologique sur animal, 81 des 130 réactions positives étant lisibles après incubation de 24 heures. Nous conseillons de l'employer.

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